

Protein-Membrane Interaction Is Essential to Normal Assembly of the Microsporidian Spore Invasion Tube

EARL WEIDNER^{1,2}, S. B. MANALE^{1,2}, S. K. HALONEN^{1,3}, AND J. W. LYNN¹

¹Department of Zoology and Physiology, Louisiana State University, Baton Rouge, Louisiana 70803;

²Marine Biological Laboratory, Woods Hole, Massachusetts 02543; and ³Department of Neurology, Albert Einstein College of Medicine, Bronx, New York 10461

Abstract. Changes in the protein-membrane interaction during assembly of the microsporidian spore invasion tubes were followed by electron microscopy, by video imaging with differential interference contrast (DIC), and by the fluorescent probes 4',6-diamidino-2-phenylindole (DAPI) and 9-diethylamino-5H-benzo[α]phenoxazine-5-one (Nile red). Microsporidian spore invasion tubes form by the eversion of polar filament protein (PFP) and presumptive extrusion apparatus (EAP) membrane. Both of these components are essential for formation of the invasion tube. The results indicate that the behavior of the EAP membrane is greatly affected by the position and chemical state of the PFP at the eversion area that constitutes the advancing tube terminal assembly site (TAS). Visual evidence indicates that the EAP membrane is the vehicle for PFP and that this membrane also provides the envelope that surrounds the sporoplasm after its passage through the invasion tube.

Introduction

Microsporidians are a large group of intracellular parasites widely distributed within many animal groups (Canning and Lom, 1986). Some species, as opportunistic parasites, have developed a significant presence in AIDS patients (Ornstein *et al.*, 1990; Canning and Hollister, 1991; Cali *et al.*, 1993). The spore stage is characterized by an extrusion apparatus (EAP) with two principal assemblages, a polar filament protein (PFP) coil, and an envelope consisting of an extensive pleated system of

membranes (Cali, 1991). The infective microsporidian spore is remarkable because it discharges its entire contents (excluding the plasma membrane) through a 100-nm-wide tube and into a target cell with the time frame of 100–150 ms (Lom and Vavra, 1963; Undeen, 1990; Frixione *et al.*, 1992). During spore discharge, the invasion tube cinematographically resembles an extruding cnidarian nematocyst (Blanquet, 1983), a discharging capsule from a myxosporean spore (Lom and Noble, 1984; El-Matbouli *et al.*, 1992), or a discharging toxicyst from the ciliate *Loxophyllum* (Hausmann, 1978). In all of these extreme forms of exocytosis, the extrusion apparatus is confined to an organelle with an outer wall; but, with microsporidians, the EAP is segregated from the spore cytoplasm by a membrane, rather than a walled structure (Weidner *et al.*, 1984).

The primary components of the microsporidian extrusion apparatus, the PFP and the EAP membrane, display a significant interactive change during invasion tube discharge. In the presence of PFP, the EAP membrane initially develops into a tubular shape; but with removal of the PFP, the membrane develops a saccular shape. The PFP is a low molecular weight peptide (recently, resolved to two elements) that assembles into stable polymers. Polymeric PFP is stable in sodium dodecyl sulfate but dissociates in dithiothreitol after urea pretreatment (Weidner, 1976). The fluidity of polymerized PFP is modulated by Ca^{2+} and by pH yielding unstable monolayers (Weidner, 1976, 1982).

Here, we report that the EAP membrane is essential for the formation of the invasion tube because it affects the assembly of PFP. Major modulations in the interactions between the PFP and the EAP membrane occur at the advancing tube tip terminal assembly site (TAS). Video imaging with differential interference contrast

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Abbreviations Nile red, 9-diethylamino-5H-benzo[α]phenoxazine-5-one; DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast; EAP, extrusion apparatus; PFP, polar filament protein; TAS, terminal assembly site.

(DIC) and fluorescent probes were used in this study. 4',6-diamidino-2-phenylindole (DAPI), a binder of certain polyanionic substrates and double-stranded DNA (Bonne *et al.*, 1985; Meszaros *et al.*, 1987), was used to identify the nuclear and cytosol positions during invasion tube discharge. A lipophilic generalized membrane probe, 9-diethylamino-5H-benzo[α]phenoxazine-5-one (Nile red) (Greenspan *et al.*, 1985; Greenspan and Fowler, 1985), was used to monitor the membrane because it provides an intense fluorescence and did not affect spore discharge. A preliminary study using fluorescent probes to monitor spore discharge has been previously published (Weidner *et al.*, 1994).

Materials and Methods

Microsporidian spores

Spraguea lophii spores were recovered from the central nervous system of the monkfish, *Lophius americanus*. Monkfish were provided by the Marine Resources department of the Marine Biological Laboratory in Woods Hole, Massachusetts. Spores were purified by a wash cycle described elsewhere (Weidner, 1976).

Spore activation

Isolated spores previously maintained in 0.05 M Hepes were placed in medium adjusted to pH 7 with 10^{-5} M Ca^{2+} for 30 min and then transferred into activation medium consisting of 2.0% mucin (human or porcine type 1 from Sigma) and Hepes buffer adjusted to pH 9.5.

Fluorescence

S. lophii spores were placed in incubation medium containing 0.1 $\mu\text{g}/\text{ml}$ 4',6-diamidino-2-phenylindole (DAPI) or 0.1 $\mu\text{g}/\text{ml}$ 9-diethylamino-5H-benzo[α]phenoxazine-5-one (Nile red) in 0.05 M Hepes buffer for 30–45 min. Spores were then transferred to activation medium and examined with a Nikon Microphot FXA or Zeiss Axio-phot optical system. The DAPI imaging was carried out with a 365-nm excitation frequency and with a 400-nm highpass barrier filter. Nile red imaging was carried out using an FITC Filter set (Nikon) with a 480-nm excitation frequency and a 510-nm highpass barrier filter. Images were examined with 60 \times or 100 \times Fluor or Planapochromatic oil immersion lenses.

Video

Images of microsporidian spore discharge were recorded using a customized Nikon Optiphot-2 adapted to an Odyssey X11 laser confocal unit (Noran Instruments, Inc.). Video recordings were recovered with a

Nikon 60 \times oil immersion lens (Nikon Inc., Melville, NY). Confocal fluorescence measurements were recorded using a 488-nm excitation wavelength and a 515-nm highpass barrier filter. Image recordings were saved on an optical memory disk (Panasonic 2028F), digitalized, and analyzed with Intervision (Noran Instruments, Inc.). Photographs were taken using a 35-mm camera to capture images from a 23-in silicon graphics monitor.

Electron microscopy

Negative-stained material was prepared by applying discharged spores onto Formvar-coated grids. Grids were stained with 2% phosphotungstic acid adjusted to pH 6.9. For topographical images of discharged spores, platinum-carbon replicas were prepared as described earlier (Koo *et al.*, 1973). Material prepared for sectioning was fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer, post-fixed in osmium, and processed by standard procedures (Weidner, 1976).

Results

Transmission electron microscopy of microsporidian spore invasion tubes

Polar filament protein (PFP) and membrane assembly were the main part of the extrusion apparatus of *S. lophii* spores. Before filament extrusion and tube formation, para-crystalline-like proteins organized as a coil at the polar end and extended to a subsurface spore aperture (Fig. 1A). In unfired spores, a single membrane surrounded the filament coil and extended as a system of pleats along with the ascending filament near the polar aperture. However, with spore discharge, PFP emerged from the aperture and became positionally reversed in reference to the filament membrane (Fig. 1A, B, and C). The discharged membrane was a double cylinder at the distal region of the emerging tube with PFP apparent within the membrane cylinders (Fig. 1B and C). However, the extruded tubes showed significant amounts of PFP on the membrane exterior assembled as a stable outer envelope (Fig. 1C). Platinum-carbon replicas of discharged tubes show a distinction between empty, incompletely extruded, and fully extruded invasion tubes. The incompletely extruded tubes (50 μm or less) had internal cylinders of material (Fig. 1D), whereas the fully extruded invasion tubes (measuring 65–70 μm) were devoid of internal cylinders (Fig. 1D).

DAPI and DIC imaging of discharging S. lophii spores

The DNA fluorophore, DAPI, labeled *S. lophii* nuclei without significantly affecting spore activation and tube discharge when incubations were for less than 1 h with

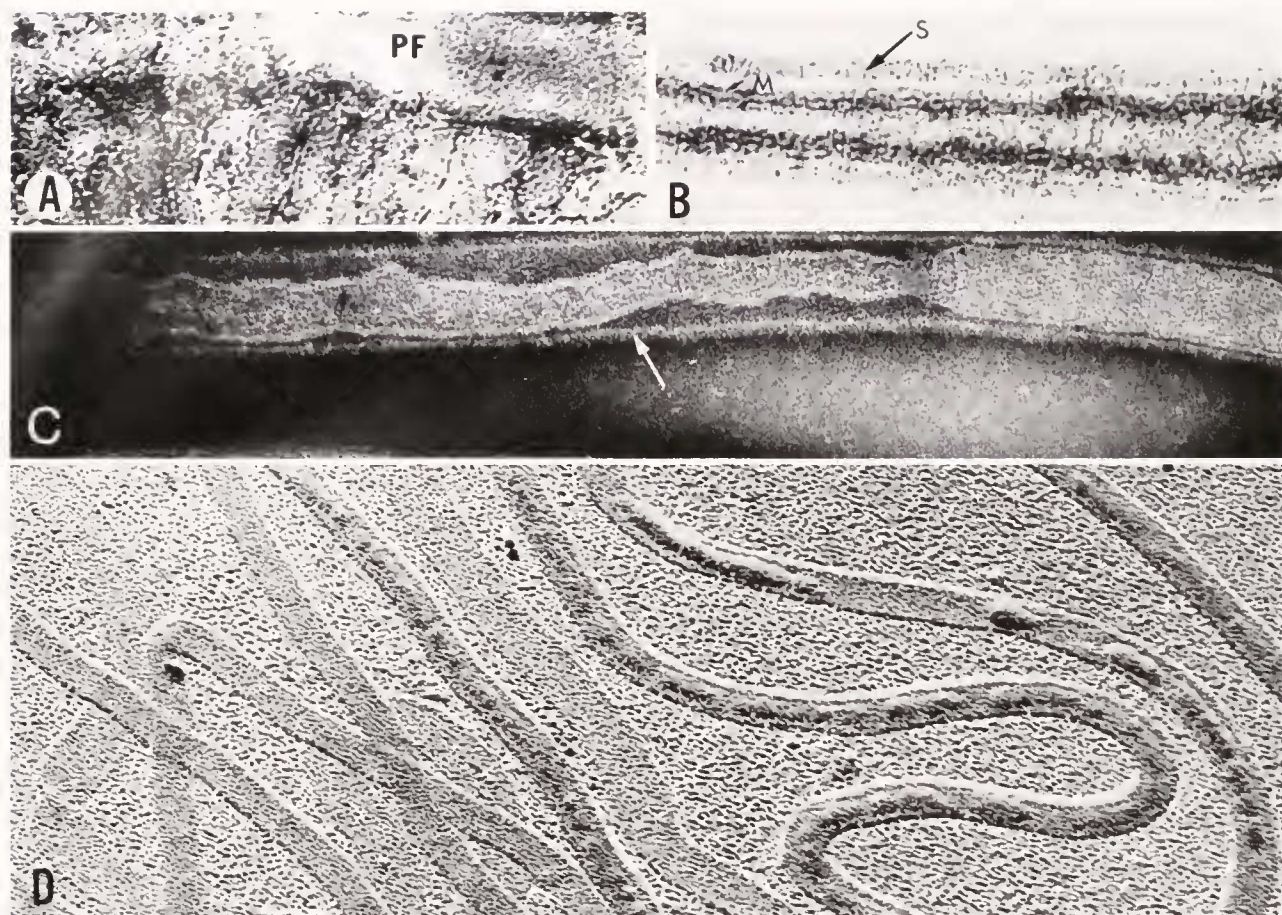


Figure 1. Electron micrographs of microsporidian extrusion apparatus. (A) Unfired spore of *Spraguea lophii* with ascending part of polar filament protein (PFP) surrounded by membrane pleats. (B) Partially discharged spore is shown in section: the double cylinders of membrane are apparent (M), as is the exteriorized PFP (S). (C) Negative-stained invasion tube shows outer protein envelope (arrow) and an inner membrane cylinder bearing what appears to be PFP material. (D) The platinum-carbon replica distinguishes between extruded invasion tubes with inner cylinders of membrane (incomplete), and completely extruded tubes that appear empty.

5 μ M of label. However, extended (overnight) incubations with 50 μ M DAPI affected tube extrusion and sporoplasmic sac emergence at the end of the tube. When DIC was coupled with DAPI fluorescence, nuclear movement was observed in association with invasion tube formation and sporoplasmic sac emergence (Fig. 2A–F). Sporoplasmic sacs did not appear at the tube ends until the tubes had reached a maximum length of 70 μ m. The spore nucleus did not begin to traverse the tube until 90–95% of the discharged tube had emerged from the spore. However, in 1–2% of the discharged spores with extruded sporoplasmic sacs, the nuclei were still within the invasion tube (Fig. 2D). The failure rate of sporoplasmic sac emergence or of sporoplasm extrusion was increased with extended spore incubations (6–12 h) in higher concentrations of DAPI (50 μ M).

Video imaging of microsporidian spore invasion tube discharge with Nile red

Intense Nile red fluorescence was apparent during membrane discharge through invasion tubes, but the intensity diminished rapidly after the sporoplasmic sacs had emerged (Fig. 3A and B). Similarly, discharged tubes pre-washed in SDS detergent showed little fluorescence. Because of the intensity of Nile red fluorescence for membrane, this dye was used to monitor the movement of membrane down the invasion tube during spore extrusion (Fig. 4A–F). Video analyses showed that at the onset of spore discharge, the Nile red fluorescence was highest at the distal end of the emerging tube (Fig. 5A–F). Although the fluorescence was strongest along the distal 15 μ m of invasion tubes 50–55 μ m in length, fully extruded invasion tubes (70 μ m) exhibited an even fluorescence

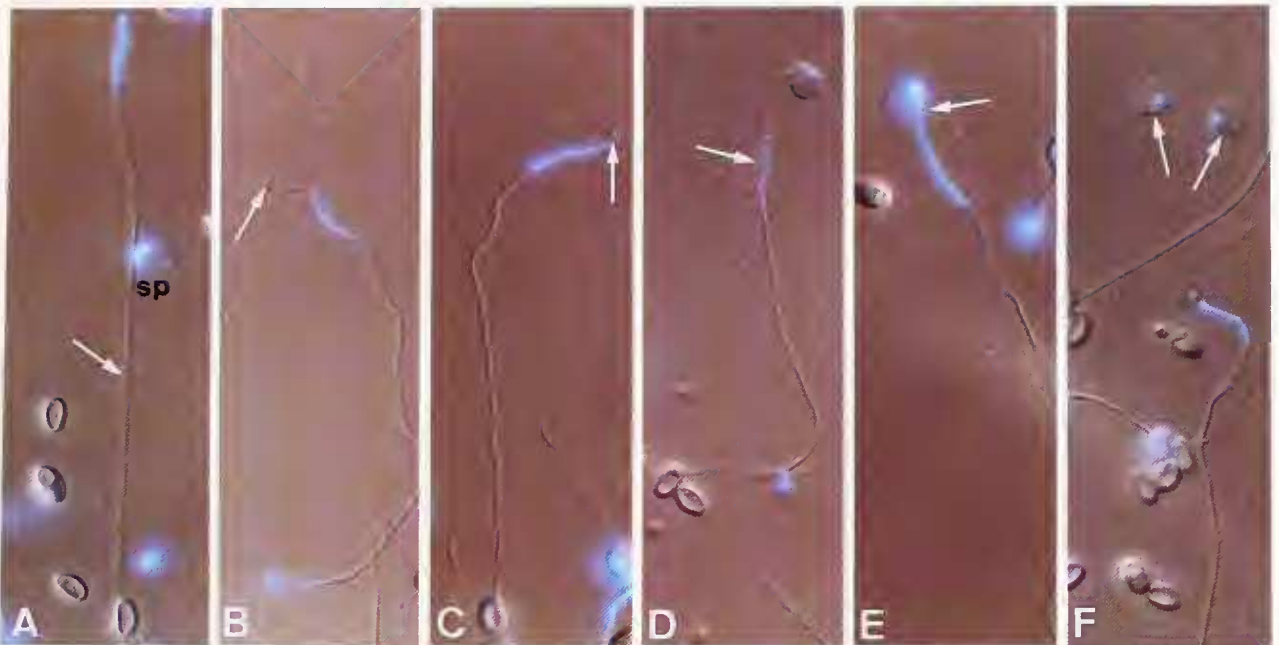


Figure 2. Light optics showing discharging *Spraguea lophii* spores prelabeled with DAPI and examined with DIC imaging. (A) The discharged spore invasion tube has emerged (arrow) with the nucleus (DAPI-blue stain) at the tube tip. Note the fully emerged sporoplasm (sp) from another spore. (B and C) Sporoplasmic sac emerging at the invasion tube tip (arrows). (D) The sporoplasmic sac has fully emerged, but the nucleus (arrow) is still within the invasion tube. (E) Nucleus is emerging into the sporoplasmic sac at the invasion tube tip. (F) Fully emerged sporoplasm with nuclear components (arrows).

throughout the tube (Fig. 5G). Ultrastructural observations indicate that discharged tubes 40–50 μm in length had double membrane cylinders at the distal ends; however, fully extruded invasion tubes did not exhibit double membranes. Tube fluorescence was reduced significantly with the emergence of the sporoplasmic sac membrane at the tube ends (Figs. 4F, 5G and H). Ultrastructural observations of discharged spores with extruded sporo-

plasmic sacs revealed discharged tubes without membranes.

DIC imaging of microsporidian invasion tubes immediately prior to sporoplasmic sac emergence

Ultrastructural studies indicate that the invasion tube tips had double membrane cylinders at the emerging tube

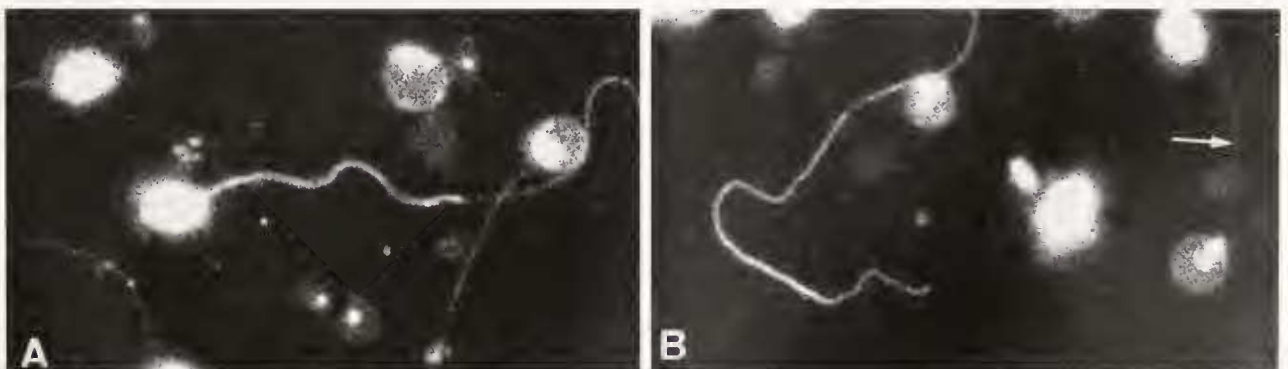


Figure 3. Light optics showing discharging *Spraguea lophii* prelabeled with Nile red. Note the intensity of the fluorescence in the incompletely discharged tubes, indicating the presence of the membrane cylinders extruding from the discharge apparatus (A and B). Arrow shows a completely discharged invasion tube that has been cleared of the membrane cylinders; fluorescence is nearly absent. The spherical body at the tip of this invasion tube is the sporoplasmic sac (arrowhead).

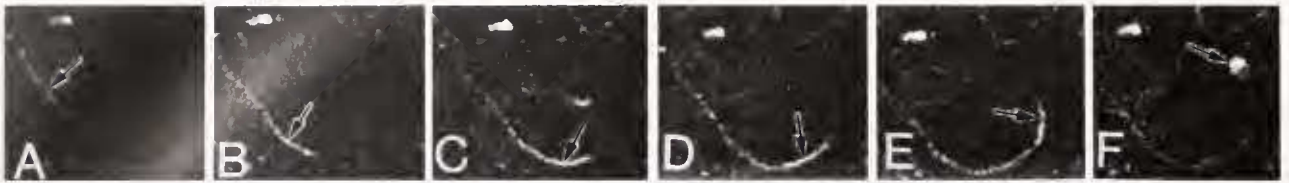


Figure 4. Video sequence showing Nile-red-labeled *Spraguea lophii* spore undergoing tube discharge. (A–E) The fluorescence is higher at the tube tip; ultrastructural images show this area to have two membrane cylinders. (F) Tube is completely assembled and the membrane has emerged into a sporoplasmic sac. Arrows show the end of the extruding tube (A–E) and sporoplasm (F).

ends. With the completion of tube assembly (65–70 μm), membrane sacs emerged followed by a flow of sporoplasm into the sacs. We used DIC imaging to investigate the nature of the tube tip immediately before sporoplasmic sac emergence. Assuming that the inner membrane cylinder moved by an everting motion over the surface of the external cylinder of membrane at the tube tip, a predictable funnel-shaped bladder would be expected before full sporoplasmic emergence. Such a funnel-shaped emergence was observed milliseconds before the appearance of the full sporoplasmic sac (Fig. 6A–C). The timing for this event was significantly increased by preincubating spores in 50 μM DAPI overnight. This did not affect spore activation, but produced either a block or a delay in sporoplasmic sac emergence at the tube end.

Discussion

Interactions of invasion tube protein and membrane during assembly

Nile red video data indicate a strong fluorescence for membrane along the length of the invasion tube throughout tube assembly; however, this fluorescence moves to the assembled tube end with the emergence of the sporoplasmic sac. Ultrastructural observations indicate that tube fluorescence is due to the double membrane cylinders that extend the length of forming tubes. Before the PFP begins the eversion process with membrane at the advancing tube terminal assembly site (TAS), the protein is believed to be monomeric and confined to the inside of the tube membrane. However, when the PFP begins eversion with membrane at the TAS, evidence indicates that the protein transforms into a stable polymer (Weidner, 1976). Ultrastructural observations of the TAS indicate that the PFP polymer and adjoining membrane are continuous with the inner tube monomeric PFP and inner membrane at the TAS. At the opposite end of the invasion tube from the TAS, the polymerized PFP and the external cylinder of membrane are attached to the polar aperture of the spore. This membrane has a short-term attachment restricted to the period of tube assembly as indicated by ultrastructural and fluorescent probe studies showing that

this membrane is liberated and discharged during sporoplasmic sac formation at the end of the invasion tube.

Video analysis of discharging spores shows invasion tubes growing exclusively at the TAS. This is most obvious in discharging tubes that follow a tortuous path, since all points on the formed tube remain fixed in position while the TAS movement continues by eversion (Weidner, 1982). Furthermore, when video recording data of tube discharge were analyzed with digital sequential frame subtractions (30-ms intervals), the only apparent change was at the discharging tube tip, indicating membrane movement exclusively at the TAS. Finally, DIC imaging clearly indicates tube growth at the TAS by eversion, especially after completion of tube assembly when the membrane at the TAS begins eversion by forming a funnel-shaped bladder (as modeled in Fig. 8).

Membrane behavior in the microsporidian invasion tube is affected by PFP

When membrane assemblages move on or within a cell, the shape the membrane assumes depends on the interactions between the membrane and the associated proteins (Cunningham and Edelman, 1990). The question of whether this general rule applies to specialized organelles that explosively discharge membrane by eversion has arisen because a tubular shape was thought to be an inherent property of this membrane configuration. However, specific examples indicate that this is not the case. Certain kinds of nematocysts (rhopalomes of siphonophores and spirocysts of anemones) evert a saccular membrane rather than a slender tubular one (Hyman, 1940). Although the end result of microsporidian spore discharge is membrane that is saccular in shape, the initial process begins with the eversion of membrane in a slender tube. Assembly of the microsporidian spore invasion tube continues until all the PFP has emerged to complete tube assembly. After all the PFP has emerged at the TAS, membrane continues to exit the tube. Since this membrane is without associated PFP, it shows an altered behavior by forming a saccular shape at the TAS.

Evidence indicates that the PFP effect on membrane depends on its position and chemical state within the ex-

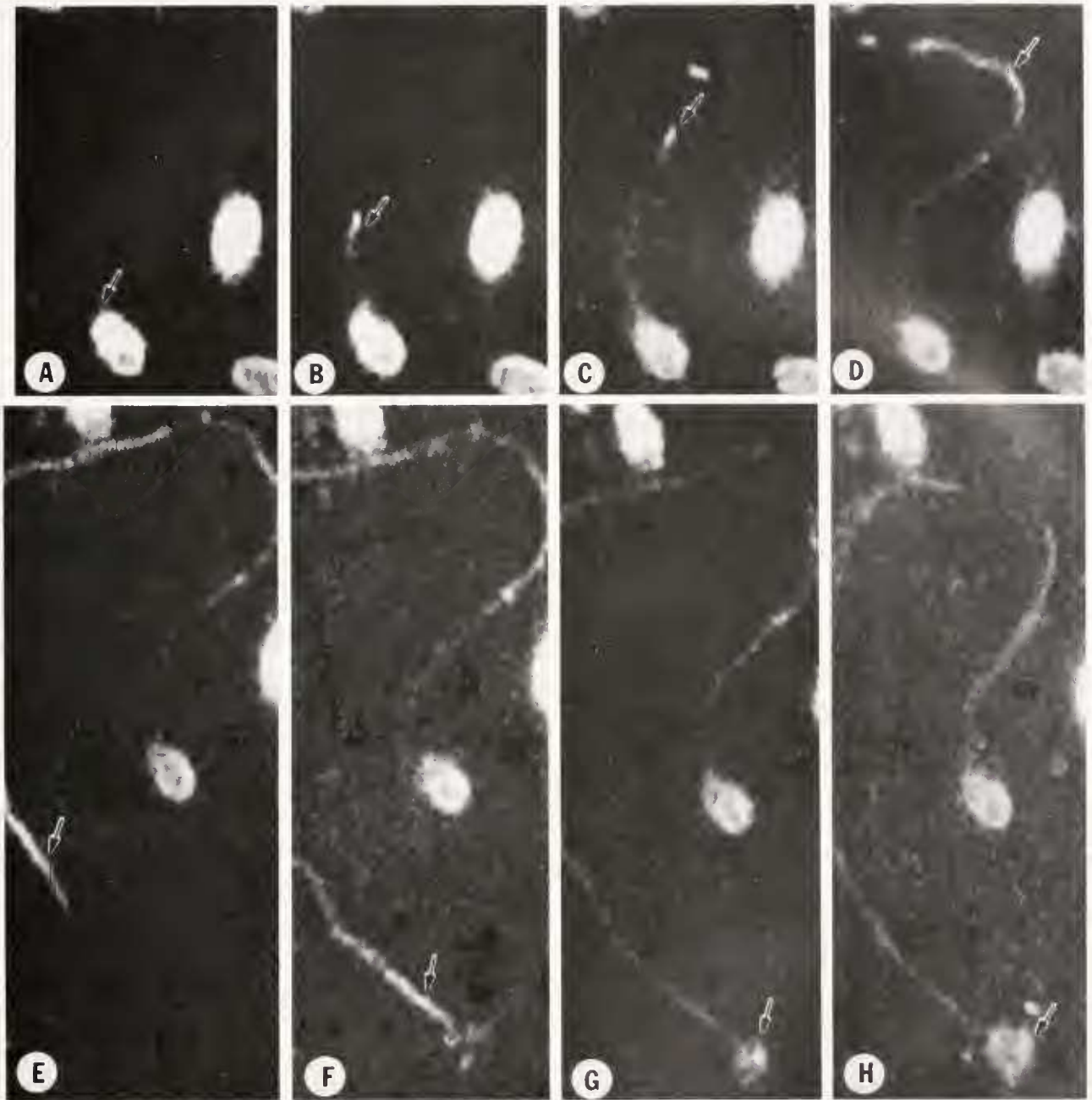


Figure 5. Digital sequential frame subtractions showing Nile-red-labeled *Spraguea lophii* spore undergoing tube discharge and sporoplasmic sac emergence. Each image represents a 30-ms shift in the video. (A-F) The invasion tube is emerging, with the highest level of membrane fluorescence at the tube tip (arrows). (G and H) Fluorescence is greatest in the extruded sporoplasmic sac (arrows), while the remaining tube has lost much of the fluorescence.

trusion tube. During tube assembly, the PFP first appears fluid and has an apparent adhesive affinity for membrane, as indicated by the sharpness by which the PFP everts with its adjoining membrane at the TAS. The sharpness of eversion at the TAS is believed to give the tube some capacity to pierce through substrate (Lom and Vavra,

1963), an ability that would be essential to penetration of host-cell membrane. Although the fluidity of the PFP within the invasion tubes is not clear, isolated PFP polymer displays increased fluidity when subjected to $10^{-4} M$ Ca^{2+} ; moreover, discharged PFP tubes show some tendency to fuse or branch when exposed to medium with

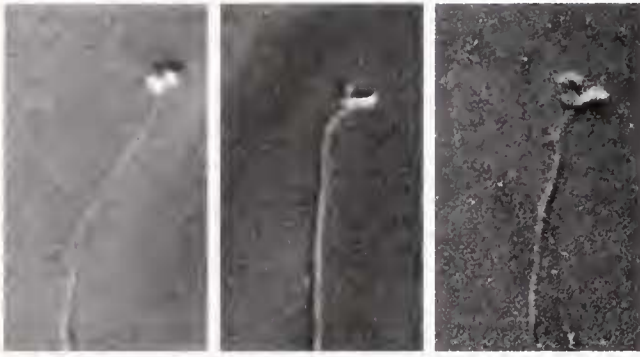


Figure 6. DIC imaging of microsporidian spore invasion tubes immediately before sporoplasmic sac emergence. These three images show a characteristic funnel-shaped bladder emerging from the tube tip milliseconds before the full sporoplasmic sac emerges. Such a shape is predicted when an inner cylinder of membrane is everting and sliding over an outer envelope. Tube thickness is 0.1–0.13 μm

calcium (Weidner, 1982). Also, isolated PFP displays significant fluidity under *in vitro* conditions, although it will not form discrete tubular profiles (Weidner, 1976). When the PFP emerges from discharging tubes at the TAS, the PFP is clearly a polymer, and its stability is obvious because it resists shape change across the pH range and because it resists dissociation in 1% SDS. On the other hand, internal tube PFP that has yet to reach the TAS appears to dissociate in SDS (Weidner, 1976), indicating that it is not a polymer, but monomeric. When monomeric PFP approaches the TAS, it apparently retains an adherence for membrane since these two elements always exteriorize together at the TAS regardless of the physical conditions of the external medium. Several observations indicate that polymerized PFP has little or no membrane adhesiveness. Ultrastructural observations indicate that a substantial space is present between exteriorized PFP polymer and the adjoining membrane within the invasion tube. Also, after all the internal PFP has exited the tube, the remaining membrane of the tube is liberated from the attachment point at the polar aperture and passes to the sac at the end of the tube. Therefore, it appears that monomeric PFP has a strong affinity for membrane until it is exteriorized from the invasion tube, where it becomes a polymer and loses some of its membrane affinity. In a sense, PFP is a morphoregulatory molecule with the membrane behavior being regulated by its association with protein (see Cunningham and Edelman, 1990).

Plasma membrane origins during microsporidian spore discharge

One remarkable feature of microsporidian spore discharge is the discard of the plasma membrane with the spore ghost while all of the sporoplasm (nucleus and cy-

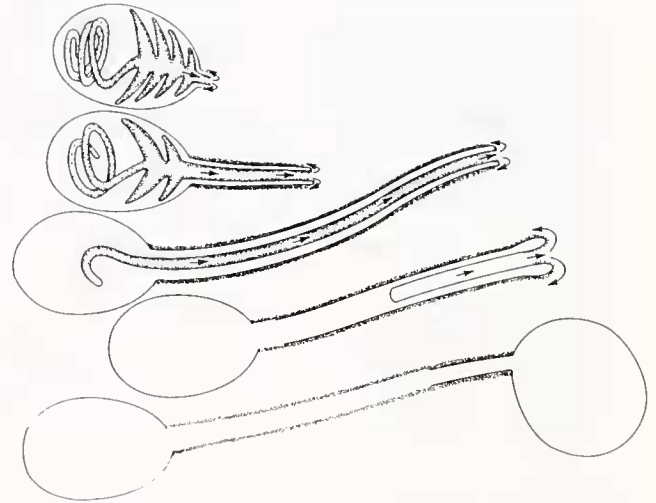


Figure 7. Model illustrating the emergence of the extrusion apparatus (EAP) from the spore. The membrane-bound polar filament everts, with membrane sliding on the membrane, as the inner cylinder delivers the polar filament protein (PFP) (dots within the apparatus) to the tube tip, and the protein stabilizes into outside polymer. Ultimately, when all the protein has been assembled, the remaining membrane continues to slide out and contributes to the sporoplasmic sac at the end of the tube. The last diagram in the sequence shows the empty tube, now represented only by the PFP polymer, and the emerging sporoplasmic sac. The sporoplasm characteristically enters the sporoplasmic sac milliseconds after its emergence.

toplasm) exits through a tube and into a new membrane sac. This discharged sac is thought to develop by the eversion of the extrusion apparatus (EAP) membrane. The EAP membrane is the only probable source for the sporoplasmic sac membrane (Weidner *et al.*, 1984). The model proposed here illustrates how the sporoplasm nucleus and cytoplasm are transferred out of the spore by membrane eversion of the EAP (Figs. 7 and 8). The movement of sporoplasm into the sporoplasmic sac generally appears to occur concurrently. Whether any spo-

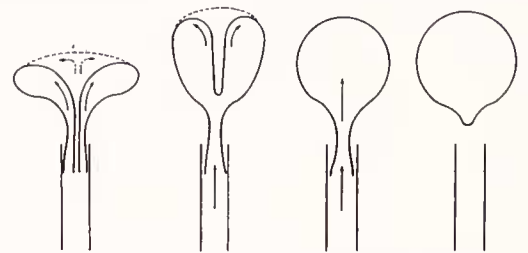


Figure 8. Scheme illustrating how the inner cylinder of membrane slides over the outer cylinder during the emergence of the sporoplasmic sac at the end of the discharged invasion tube. Such an event happens only when the discharged tube is fully assembled. This scheme is primarily based on the DIC images of the tube tip (see Fig. 6) in addition to the ultrastructural observations.

roplasm moves into the sporoplasmic sac without membrane is not clear at this time. The following observations with DAPI and DIC imaging indicate that the EAP membrane system is exterior to the nuclear and cytosolic components of the spore. First, the nucleus and cytosol do not enter the invasion tube until it is fully assembled. Because the invasion tube forms by everting membrane from the EAP, its membrane system segregates it from the sporoplasm within the spore. Thus, the EAP membrane probably has to turn itself inside out before the sporoplasm can move into the EAP compartment. Second, discharged, empty EAP membranous sacs are occasionally observed attached to the ends of invasion tubes while the DAPI stained nucleus remains within the tube. This condition would be impossible unless the EAP and sporoplasm were initially segregated within the spore. The most logical explanation is that the EAP membrane provides the new plasma membrane for the discharged sporoplasm through an everting process.

Figure 8 illustrates our model for EAP eversion into the plasma membrane sac. It is based on DIC images of the TAS immediately preceding full emergence of the membrane sac. The funnel-shaped bladder that commonly appears at the end of the tube is believed to be derived from the inner cylinder of the EAP membrane moving in relation to the outer envelope. Prolonged incubations of activated spores with DAPI slowed the rate of membrane emergence at the ends of assembled tubes and made it easier to observe sac formation. Extended DAPI incubations are thought to affect the membrane movement by perturbing the Ca^{2+} levels within the tube. PFP assembly and disassembly are affected by Ca^{2+} (Weidner, 1982) and, because DAPI can affect the kinetics of Ca^{2+} uptake or release in molecular systems (Meszaros *et al.*, 1987), the changes in membrane behavior caused by prolonged incubations in DAPI may be due either to direct interference with the PFP or to alterations in Ca^{2+} uptake that change the PFP polymerization.

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